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# Antithrombotic Activity of Sunghyangjunggisan

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**Abstract** – As apart of our continuing search for antistroke agents from the herbal medicinal resources, we examined *in vitro, ex vivo* and *in vivo* the possibility of Sunghyangjunggisan and its ingradients as a novel antithrombotic agent. *In vitro* ADP- and collagen-induced rat platelet aggregations were potently inhibited by Arisaematis Rhizoma, Cinnamomi Cortex and Zingiberis Rhizoma in a dose-dependent manner, but not by Sunghyangjunggisan. However, Sunghyangjunggisan significantly inhibited *ex vivo* rat platelet aggregation. Arisaematis Rhizoma, Atractylodis Rhizoma Alba, and Pinelliae Rhizoma also significantly inhibited *ex vivo* rat platelet aggregation. Sunghyangjunggisan, Alpiniae Fructus and Zingiberis Rhizoma showed significant protection from death due to pulmonary thrombosis in mice. Therefore, Sunghyangjunggisan can express the antithrombotic action, when it is orally administered.
**Keywords** – Sunghyangjunggisan, stroke, antithrombosis

## Introduction

Platelets play an important role in the pathogenesis of thrombosis. The interactions between the platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases, such as myocardial infraction stroke, and arteriosclerosis (Mustard, 1975; Mustard and Packham, 1990; Dinerman and Mehta, 1990). Once the blood vessels are damaged, platelet aggregation occurs rapidly to form hemostatic plugs or arterial thrombi at the sites of vessel injury or in regions where the blood flow is disturbed. These thrombi are the source of thromboembolic complications of arteriosclerosis, heart attacks, stroke, and peripheral vascular disease (Packham, 1994; Stein and Fuster, 1989; MacMahon and Fuster, 1989). Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis. A number of antiplatelet herbal medicines and their polyprescriptions have been evaluated for their effects in preventing the development of thrombosis or its recurrence.

As a part of our continuing search for biological active antistroke agents from medicinal resources, we investigated Sunghyangjunggisan because it has been used frequently for patients who suffer from stroke in Korea (Bae *et al.*, 1987). In the present study, we examined the possibility of Sunghyangjunggisan and its ingredients as a novel antithrombotic agent by determining its inhibitory effect on platelets induced by various aggregating agents *in vitro* and *ex vivo*, and its antithrombotic effect *in vivo*.

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## Materials and Methods

**Materials** – Adenosine 5'-diphosphate (ADP), epinephrine, collagen, bovine serum albumin, prothrombin, thromboplastin, thrombin and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The other chemicals were of analytical reagent grade.

**Plant materials and extraction** – The herba of *Pogostemon cablin*, the herba of *Perilla frutescens*, the rhizome of *Arisaema amurense*, the rhizome of *Aucklandia lappa*, the rhizomes of *Atractylodes macrocephala*, the pericarpium of *Citrus unshiu*, the rhizome of *Pinellea ternata*, the pericarpium of *Citrus unshiu*, the pericarpium of *Areca catechu*, the cortex of *Cinnamomum cassia*, the rhizoma of *Zingiberis officinale*, the fructus of *Alpinia oxyphylla*, the radix of *Glycyrrhiza uralensis*, the fructus of *Zizyphus jujuba* were in Korea. They were purchased from Kyungdong Crude Drug Market, Soul, Korea, 1999. They were identified by N-J Kim (East-West Medical Research Institute, Kyung Hee University) and voucher specimens (990702-9 990702-23) were kept at College of Pharmacy, Kyung Hee University. One hundred grams of each herbal medicine (100 g) or

**Table 1.** Composition of the ingredients of Sunghyangjunggisan

| Herbal medicine | Composition (g) | Voucher Specimen |
|---|---|---|
| Pogostemi Herba | 6 | KHP990702-9 |
| Perillae Herba | 4 | KHP990702-10 |
| Arisaematis Rhizoma | 4 | KHP990702-11 |
| Saussureae Radix | 4 | KHP990702-12 |
| Atractylodis Rhizoma Alba | 2 | KHP990702-13 |
| Aurantii nobilis Pericarpium | 2 | KHP990702-14 |
| Pinelliae Rhizoma | 2 | KHP990702-15 |
| Aurantii Immatri Pericarpium | 2 | KHP990702-16 |
| Arecae Pericarpium | 2 | KHP990702-17 |
| Cinnamomi Cortex | 2 | KHP990702-18 |
| Zingiberis Rhizoma | 2 | KHP990702-19 |
| Alpiniae Fructus | 2 | KHP990702-20 |
| Glycyrrhizae Radix | 2 | KHP990702-21 |
| Zingiberis Rhizoma | – | KHP990702-22 |
| Zizyphi Fructus | – | KHP990702-23 |
| Sunghyangjunggisan | 36 | |

Sunghyangjunggisan (Table 1) were extracted twice with 500 ml of boiling water. After evaporation, each extract was used for the study.

**Animals** – Male Sprague-Dawley rats (male, 180-220 g) and ICR mice (male, 20-24 g) were purchased from Sam Yook Animal Co. (Korea) and acclimatized for one week at a temperature of 22±1°C and a humidity of 55±5% with free access to a commercial pellet diet obtained from Samyang Co. (Korea) and drinking water before the experiments. The animal experiments were carried out in accordance with international guidelines.

**Preparation of platelets** – Blood was collected from the rats by cardiac puncture into a plastic flask containing 2.2% sodium citrate (1:9 v/v). The platelet rich plasma (PRP) was prepared by centrifuging the blood at 120×g for 10 min, and then again at 850×g for 20 min to prepare the platelet poor plasma (PPP) (Teng and Ko, 1988).

**Assay of *in vitro* antiplatelet aggregation activity** – The platelet aggregation was measured by turbidometry using a dual channel Whole Lumini-Ionized Calcium Aggregometer (Chrono-Log Co., Ltd, Havertown, PA, USA) according to the method of Born and Cross (1963). Briefly, rat PRP (300 μl) was incubated at 37°C for 2 min in the aggregometer with stirring at 1200 rpm, and then stimulated with ADP and collagen. The herbal medicines or aspirin (as a reference agent) were incubated with PRP for 3 min, followed by addition of the aggregation agents. Any change in light transmission was recorded for 10 min after stimulation with these agents.

**Assay of *ex vivo* antiplatelet aggregation activity** – Male SD rats were used after overnight fasting. One gram of herbal medicine extract (or 50 mg/kg of aspirin) per kg body weight was administered orally to the rats for three days. The blood was collected 3 h after the final sample treatment, and the PRP was previously described. Platelet aggregation was induced by 80 μg/ml of collagen or 8 μM of ADP. The antiplatelet activities of the sample were investigated according to the method of Kimura *et al.* (1985).

**Assay of *in vivo* antithrombotic activity** – The antithrombotic effects of herbal medicines were investigated by the mouse thromboembolism test according to the method of DiMinno and Silver (1983). Male ICR mice were used after overnight fasting. The herbal medicines (1 g/kg), or aspirin (50 mg/kg) as a positive control, and 0.5% CMC solution were administered orally. A mixture solution of collagen (110 μg) and epinephrine (13 μg) was injected into the mouse tail vein 90 min after the sample treatment, and pulmonary thrombosis was induced 3 h after oral administration of the samples. The number of dead or paralyzed mice was recorded for up to 15 min, and the percentage of protection was calculated as follows: [1-(dead + paralyzed) / total]×100

**Tail bleeding time in conscious mice** – The bleeding time was measured as described by Hornstra *et al.* (1981). The bleeding time is designed to determine the blood's ability to form a hemostatic plug, in which the platelet, plasma factor, and blood vessel wall are involved. In short, herbal medicines (1 g/kg) or aspirin (50 mg/kg) and the 0.5% CMC solution were administered once a day for three days. At the 3 h after the oral administration of the samples, the tail of the male ICR mouse was transected 2 mm from the tip, and 1.5 cm of the distal portion was vertically immersed in saline at 37°C.

**Assay of *in vitro* coagulation parameters** – The plasma clotting times, activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were measured by a modification of Hara's method (1994). The PPP was incubated with the samples for 7 min at 37°C, and the coagulation was started with by adding $CaCl_2$, 100 μl of thromboplastin, and 100 μl of bovine thrombin into the 100 μl of incubated plasma for the APTT, PT and TT assays, respectively.

## Results

***In vitro* antiplatelet aggregation effect** – The *in vitro* inhibitory activity of Sunghyangjunggisan and its ingredients on platelet aggregation was measured (Table 2). Cinnamomi Cortex and Zingiberis Rhizoma, which are ingredients of this polyprescription, also potently inhibited ADP-induced rat platelet aggregation *in vitro*, with $IC_{50}$ values of 0.8 and 1.0 mg/ml, respectively. Arisaematis Rhizoma and

**Table 2.** Effect of Sunghyangjunggisan and its ingredients on *in vitro* antiplatelet aggregation (aggregation inducer, ADP)

| Herbal medicine | $IC_{50}$[a] (mg/ml) | |
|---|---|---|
| | ADP | Collagen |
| Aspirin | 1.2 | 1.1 |
| Pogostemi Herba | >15 | >15 |
| Perillae Herba | >15 | >15 |
| Arisaematis Rhizoma | 1.9 | 1.5 |
| Saussureae Radix | >15 | >15 |
| Atractylodis Rhizoma Alba | >15 | 6 |
| Aurantii nobilis Pericarpium | >15 | >15 |
| Pinelliae Rhizoma | 1.5 | 3 |
| Aurantii Immatri Pericarpium | >15 | >15 |
| Arecae Pericarpium | >15 | >15 |
| Cinnamomi Cortex | 0.8 | 0.9 |
| Zingiberis Rhizoma | 6.4 | 6.5 |
| Alpiniae Fructus | 4.8 | 1.8 |
| Glycyrrhizae Radix | >15 | >15 |
| Zingiberis Rhizoma | 1 | 2 |
| Zizyphi Fructus | 4.5 | 4.1 |
| Sunghyangjunggisan | >15 | >15 |

[a]$IC_{50}$ represents a concentration showing 50% inhibition of platelet aggregation. Percent inhibition was calculated as follows: (control aggregation (%)-herbal medicine-treated aggregation (%))/ control aggregation (%) x 100 = inhibition (%).

Cinnamomi Cortex also inhibited collagen-induced rat platelet aggregation *in vitro*, with $IC_{50}$ values of 1.5 and 0.9 mg/ml, respectively. Aspirin, a reference drug that is widely used as an anti-platelet drug in clinical practice, potently inhibited ADP and collagen-induced platelet aggregation, with $IC_{50}$ values of 1.2 and 1.1 mg/ml, respectively. The antiplatelet activities of Cinnamomi Cortex and Zingiberis Rhizoma were more potent than those of aspirin.

***In vitro* antiplasma coagulation effect** – The effect of herbal medicines on plasma clotting time was evaluated by APPT, PT and TT assays using human platelet poor plasma (Table 3). Sunghyangjunggisan did not affect the tested plasma clotting times. Among its ingredients, Arisaematis Rhizoma, Pinelliae and Rhizoma Alpiniae Fructus weakly inhibited human plasma coagulation time.

***Ex vivo* antiplatelet aggregation effect** – The *ex vivo* inhibitory activities of Sunghyangjunggisan and its ingredients on platelet aggregation were measured, after they were orally administered once a day for three days into SD rats (Table 4). The inhibition of ADP- or collagen-induced platelet aggregation in the group treated with Sunghyangjunggisan was significantly higher than that of the control group. Sunghyangjunggisan inhibited ADP-induced platelet aggregation more potently than aspirin (50 mg/kg). Among its ingredients, Perillae Herba, Arisaematis Rhizoma and Pinelliae Rhizoma exhibited the most potent inhibitory activity against ADP-induced platelet aggregation. Collagen-induced platelet aggregation was potently inhibited by Atractylodis Rhizoma Alba.

**Effect on the tail bleeding time of mice** – The effect of herbal medicine on bleeding time was studied using the mouse tail bleeding system. As shown in Table 5, the tail bleeding time of the control mice was 68±12.5 s, while Sunghyangjunggisan and Alpiniae Fructus markedly prolonged the mouse tail bleeding time (>600 s) relative

**Table 3.** Effect of Sunghyangjunggisan and its ingredients on human plasma coagulation time

| Herbal medicine | Concentration (mg/ml) | Coagulation time (s) | | |
|---|---|---|---|---|
| | | APTT | PT | TT |
| Control | 0 | 36.1±1.2 | 40.8±2.6 | 31.7±2.5 |
| Pogostemi Herba | 3 | 39.4±2.1 | 23.6±1.1 | 38.1±2.4 |
| Perillae Herba | 3 | 49.1±9.8 | 30.2±1.1 | 45.9±1.5* |
| Arisaematis Rhizoma | 3 | 59.0±6.5* | 47.8±3.9* | 48.1±1.1* |
| Saussureae Radix | 3 | 34.8±4.0 | 36.1±2.1 | 38.4±0.6 |
| Atractylodis Rhizoma Alba | 3 | 28.0±6.2 | 39.6±1.1 | 34.8±0.1 |
| Aurantii nobilis Pericarpium | 3 | 28.8±7.1 | 36.3±2.1 | 33.1±3.2 |
| Pinelliae Rhizoma | 3 | 43.3±0.7* | 37.2±4.6 | 40.9±2.9* |
| Aurantii Immatri Pericarpium | 3 | 35.9±0.6 | 27.6±0.4 | 33.6±0.4 |
| Arecae Pericarpium | 3 | 33.9±2.9 | 23.6±1.1 | 32.1±1.1 |
| Cinnamomi Cortex | 3 | 37.6±0.4 | 32.3±0.1 | 35.3±0.1 |
| Zingiberis Rhizoma | 3 | 37.5±3.5 | 30.5±2.4 | 34.6±1.1 |
| Alpiniae Fructus | 3 | 45.6±3.2* | 32.0±2.7 | 41.6±1.8* |
| Glycyrrhizae Radix | 3 | 34.6±0.4 | 27.6±3.1 | 33.4±0.1 |
| Zingiberis Rhizoma | 3 | 34.6±1.1 | 32.8±5.8 | 38.9±2.2* |
| Zizyphi Fructus | 3 | 32.4±0.1 | 38.0±5.6 | 34.1±1.8 |
| Sunghyangjunggisan | 3 | 36.4±0.1 | 31.2±3.1 | 34.3±0.7 |
| Heparin | 0.003 | 174.8±22.4* | 57.0±7.7* | >500* |
| Heparin | 0.0003 | 40.6±1.0* | 48.2±4.9* | 45.9±0.6* |

The results were expressed as mean ±SD (n=3).
*Significantly different from control ($p<0.05$).

**Table 4.** Effect of Sunghyangjunggisan and its ingredients on *ex vivo* antiplatelet aggregation

| Herbal medicine | Platelet aggregation[a] (%) | |
|---|---|---|
| | ADP | Collagen |
| Control | 60.5±0.7 | 56.0±1.4 |
| Aspirin | 42.5±10.6* | 39.5±7.8* |
| Pogostemi Herba | 46±5.3* | 56.7±8.3 |
| Perillae Herba | 39.3±15.8 | 48.5±12.0 |
| Arisaematis Rhizoma | 32±19.0* | 57.7±8.4 |
| Saussureae Radix | 40.5±2.1* | 50±7.1 |
| Atractylodis Rhizoma Alba | 49.5±0.7* | 31.3±27.2 |
| Aurantii nobilis Pericarpium | 45±5.4* | 43.8±16.3 |
| Pinelliae Rhizoma | 36±7.9* | 31±24.8* |
| Aurantii Immatri Pericarpium | 49±8.5 | 58±5.7 |
| Arecae Pericarpium | 40.8±12.7* | 41±11.5 |
| Cinnamomi Cortex | 40.2±8.0* | 44.3±13.6 |
| Zingiberis Rhizoma | 51±7.1 | 34.7±25.8 |
| Alpiniae Fructus | 55±2.8 | 52±1.4 |
| Glycyrrhizae Radix | 52.5±0.7* | 39±17.0 |
| Zingiberis Rhizoma | 45±2.8* | 45.5±0.7 |
| Zizyphi Fructus | 52.5±0.7* | 50±2.8 |
| Sunghyangjunggisan | 43.8±11.6* | 26±28.4* |

Samples were orally administered once a day for three days before the test.
The results were expressed as mean±SD (n=5).
*Significantly different to control ($p$<0.05).
[a)]Platelet aggregation was induced by 80 μg/ml of collagen or 8M of ADP.

**Table 5.** Effect of Sunghyangjunggisan and its ingredients on the mouse tail bleeding time

| Herbal medicine | Dose (g/kg) | Tail bleeding time (Sec) |
|---|---|---|
| Control | Vehicle | 68±12.5 |
| Aspirin | 0.05 | 258±8.5* |
| Pogostemi Herba | 1 | 61±18.1 |
| Perillae Herba | 1 | 47±32.5 |
| Arisaematis Rhizoma | 1 | 160±87.2 |
| Saussureae Radix | 1 | 71±18.6 |
| Atractylodis Rhizoma Alba | 1 | 30±17.0 |
| Aurantii nobilis Pericarpium | 1 | 198±124.7 |
| Pinelliae Rhizoma | 1 | 285±102.6* |
| Aurantii Immatri Pericarpium | 1 | 362±211.6 |
| Arecae Pericarpium | 1 | 413±224.5* |
| Cinnamomi Cortex | 1 | 91±20.3 |
| Zingiberis Rhizoma | 1 | 550±86.0* |
| Alpiniae Fructus | 1 | >600* |
| Glycyrrhizae Radix | 1 | 173±163.7 |
| Zingiberis Rhizoma | 1 | 354±214.2 |
| Zizyphi Fructus | 1 | 63±27.4 |
| Sunghyangjunggisan | 1 | >600* |

The results were expressed as mean SD (n=5).
*Significantly different to control (0.05).

to the control (p<0.001), and was more potent than a 50 mg/kg dose of aspirin.

***In vivo* antithrombotic effect** – The *in vivo* antithrombotic activity of Sunghyangjunggisan and its ingredients was measured (Table 6). Sunghyangjunggisan showed siginificant

**Table 6.** Anti-thrombosis activity of Sunghyangjunggisan and its ingredients

| Herbal medicine | Dose (g/kg) | No. dead or paralyzed / No. tested | Protection (%) |
|---|---|---|---|
| Control | 0 | 20/25 | 20 |
| Aspirin | 0.025 | 6/10 | 40 |
| Aspirin | 0.05 | 3/10 | 70 |
| Pogostemi Herba | 1 | 4/5 | 20 |
| Perillae Herba | 1 | 4/5 | 20 |
| Arisaematis Rhizoma | 1 | 710 | 30 |
| Saussureae Radix | 1 | 4/5 | 20 |
| Atractylodis Rhizoma Alba | 1 | 5/5 | 0 |
| Aurantii nobilis Pericarpium | 1 | 4/10 | 60 |
| Pinelliae Rhizoma | 1 | 4/10 | 60 |
| Aurantii Immatri Pericarpium | 1 | 4/5 | 20 |
| Arecae Pericarpium | 1 | 4/10 | 60 |
| Cinnamomi Cortex | 1 | 5/5 | 0 |
| Zingiberis Rhizoma | 1 | 4/5 | 20 |
| Alpiniae Fructus | 1 | 2/10 | 80 |
| Alpiniae Fructus | 0.5 | 3/5 | 40 |
| Glycyrrhizae Radix | 1 | 3/5 | 40 |
| Zingiberis Rhizoma | 1 | 0/10 | 100 |
| Zingiberis Rhizoma | 0.5 | 3/5 | 40 |
| Zizyphi Fructus | 1 | 5/5 | 0 |
| Sunghyangjunggisan | 1 | 6/10 | 40 |

The samples were orally administered 90 min before tail vein injection of epinephrine and collagen.

protection from death due to pulmonary thrombosis in mice. Among their ingredients, Alpiniae Fructus and Zingiberis Rhizoma had more potent antithrombotic activity than Sunghyangjunggisan, as well as a more potent antithrombotic activity than aspirin at a dose of 50 mg/kg.

## Discussion

Sunghyangjunggisan, Chungpesagantang and Yangkyuk-sanwhatang have been used frequently in Korean Oriental Clinics for patients who suffer from stroke, but its anti-stroke activities have not been evaluated. Therefore, we investigated the antithrombotic activity of Sunghyangjunggisan and its ingredients as part of our continuing search for anti-stroke agents from medicinal resources. Sunghyangjunggisan did not show *in vitro* antiplatelet aggregation activity, although some ingredients of Sunghyangjunggisan exhibited *in vitro* antiplatelet aggregation activity. However, this Sunghyangjunggisan exhibited *ex vivo* anti-platelet activity. These results suggest that the components of herbal medicines could be converted into the active or inactive components for antiplatelet aggregation by human intestinal bacteria. For example, hesperidin, a main component of Aurantti nobilis Pericarpium, could be converted into hesperetin by human intestinal bacteria (Kim *et al.*, 1998). This compound inhibited *in vitro* platelet aggregation activity in rats (data not shown).

Cinnamomi Cortex potently inhibited plasma recalcification, followed by Glycyrrhizae Radix. Sunghyangjunggisan and most of ingredients, except Arisaematis Rhizoma, did not affect the snowstorm effect (data not shown). Sunghyangjunggisan, and some ingredients, such as Arisaematis Rhizoma, Atractylodis Rhizoma Alba, Glycyrrhizae Radix, activated urokinase, although they did not display urokinase-like activity (data not shown).

When the prolongation activity of bleeding time by Sunghyangjunggisan and its ingredients on bleeding time was investigated using the mouse tail bleeding system, Sunghyangjunggisan and Alpiniae Fructus were the most effective. These results support that the antithrombotic activity of Sunghyangjunggisan could be caused by the activities of urokinase and antiplatelet aggregation. Based on these results, we believe that antithrombic activity of Sunghyanjunggisan should be expressed by intestinal bacteria and this herbal formulae may have a potential to prevent thrombosis and cardiovascular diseases, such as myocardial infraction stroke and arteriosclerosis.

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